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Therapeutic targets in extracellular protein deposition diseases

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Abstract

Many litres of fluids are found outside cells in the human body. These fluids are rich in dissolved proteins that each have a characteristic three dimensional shape, necessary for normal function, which has been attained by the correct folding of their polypeptide chain(s). The structure of these extracellular proteins can be damaged by a variety of environmental stresses (e. g. heat and oxidation) leading to their partial unfolding and aggregation. This in turn can produce toxic soluble aggregates and/or large insoluble protein deposits, either of which can disrupt normal body function (e. g. in Alzheimer's disease and the systemic amyloidoses). A small family of abundant human blood proteins with the ability to inhibit the aggregation and deposition of stressed (partially unfolded) proteins has been discovered. These extracellular chaperones (ECs) form stable, soluble complexes with stressed proteins. It has been proposed that once bound to stressed proteins, ECs guide them to specific cell surface receptors that direct the "cargo" into lysosomes for degradation. Thus ECs and their receptors may be critical parts of a quality control system to protect the body against the deleterious effects of inappropriately aggregating extracellular proteins. This review focuses on the role of extracellular protein aggregation and deposition in disease, what little is known about mechanisms that act to control these processes, and, lastly, potential new targets for drug development. Newly identified potential drug targets include direct inhibition of protein aggregation, and manipulation of the expression levels of ECs and their receptors.

Keywords

diseases, deposition, protein, extracellular, targets, therapeutic, CMMB

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Therapeutic Targets in Extracellular Protein Deposition Diseases

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ABSTRACT

Many litres of fluids are found outside cells in the human body. These fluids are rich in dissolved proteins that each have a characteristic three dimensional shape, necessary for normal function, which has been attained by the correct folding of their polypeptide chain(s). The structure of these extracellular proteins can be damaged by a variety of environmental stresses (e.g. heat and oxidation) leading to their partial unfolding and aggregation. This in turn can produce toxic soluble aggregates and/or large insoluble protein deposits, either of which can disrupt normal body function (e.g. in Alzheimer's disease and the systemic amyloidoses). A small family of abundant human blood proteins with the ability to inhibit the aggregation and deposition of stressed (partially unfolded) proteins has been discovered. These extracellular chaperones (ECs) form stable, soluble complexes with stressed proteins. It has been proposed that once bound to stressed proteins, ECs guide them to specific cell surface receptors that direct the "cargo" into lysosomes for degradation. Thus ECs and their receptors may be critical parts of a quality control system to protect the body against the deleterious effects of inappropriately aggregating extracellular proteins. This review focuses on the role of extracellular protein aggregation and deposition in disease, what little is known about mechanisms that act to control these processes, and, lastly, potential new targets for drug development. Newly identified potential drug targets include direct inhibition of protein aggregation, and manipulation of the expression levels of ECs and their receptors.

Keywords: Extracellular protein unfolding; protein aggregation; protein deposition diseases; extracellular chaperones; receptor-mediated endocytosis; therapeutic targets**.**

INTRODUCTION

Environmental stresses can damage proteins and result in their partial unfolding, loss of function, and aggregation. In the case of extracellular proteins, these stresses can include heat, oxidation and mechanical sheer stress (e.g. encountered during the pumping of blood around the body). When a protein is exposed to physical or chemical stresses, the resulting partial unfolding typically exposes hydrophobic regions, normally buried in the folded interior of the protein, to the surrounding aqueous solvent. This is energetically unfavourable and what normally ensues is that regions of solvent-exposed hydrophobicity on neighbouring protein molecules bind to one another, leading to the formation of protein aggregates [1]. These aggregates can be toxic to cells or can increase in size to the point where they form large insoluble protein deposits, either of which can disrupt normal body function. Many different proteins undergo these same processes and there are a large number of serious human diseases for which the pathology arises from inappropriate protein aggregation [2].

Owing to the grave dangers associated with protein unfolding and aggregation, very elaborate mechanisms have evolved to "quality control" the folding state of proteins. The mechanisms that operate inside cells have been the subject of intense study for decades and are relatively well characterised [3]. In stark contrast, until recently, almost no attention had been paid to the question of how the folding state of secreted extracellular proteins is monitored or controlled. This is surprising given that inappropriate aggregation of extracellular proteins underpins the pathology of some of the most prevalent and pernicious of human diseases (e.g. Alzheimer's disease and type II

diabetes). A significant advance in this field has been provided by the discovery of a small family of abundant human blood proteins with the ability to inhibit the aggregation and deposition of stressed (partially unfolded) proteins. These extracellular chaperones (ECs), which include clusterin, haptoglobin and α_2-macroglobulin, form stable, soluble complexes with stressed proteins [4-6]. It has been proposed that once bound to stressed proteins, ECs guide them to specific cell surface receptors that direct the "cargo" into lysosomes for degradation. Thus ECs and their receptors may be critical parts of a quality control system to protect the body against the deleterious effects of inappropriately aggregating extracellular proteins [6, 7]. Identifying the elements and mechanisms of this system will open up new avenues for the development of disease therapies.

PROTEIN UNFOLDING

The vital functions that are carried out by proteins are inherently linked to their correct folding into unique three-dimensional shapes, referred to as the “native conformation”. As a consequence of the importance of adopting the native conformation, cells have extensive quality control mechanisms to monitor the progression of nascent polypeptides to mature proteins [8]. However, nascent polypeptides may irreparably misfold if RNA modification, translational amino acid misincorporation, or genetic mutations alter the primary sequence of the polypeptide. Furthermore, native proteins may partially unfold when exposed to physiologically relevant stresses resulting in misfolded conformations. Given that protein stability is collectively determined by all of the interactions contributing to the native conformation, the stability of different proteins is highly variable and may change over the lifetime of individual molecules if they become

modified. For example, damage of amino acids by reactive oxygen species (ROS) may lead to the formation of carbonyl derivatives which distort secondary and tertiary protein structure and result in partial protein unfolding, increased exposed hydrophobicity, aggregation and susceptibility to proteolysis [9]. While many antioxidant defenses exist within cells, aging and diseases such as atherosclerosis, arthritis, muscular dystrophy, cataract, pulmonary dysfunction, certain neurological disorders and some cancers are believed to be the result of free radical damage impairing cellular functions [10]. Additionally, heat stress (such as that observed in muscles during exercise) markedly increases the production of intracellular and extracellular ROS [11]. Aside from participating in reactions that produce ROS, heavy metals may disrupt protein structure by breaking internal salt bridges and disulfide bonds. Salt bridges are susceptible to interference by many compounds including acids and bases due to their ability to exchange ionic partners. Mechanical stress may also sufficiently disrupt amino acid interactions to induce protein unfolding [12].

Protein thermostability is only partly understood despite its obvious importance to all living organisms. Regardless of their relative stability all proteins may be induced to unfold providing that the kinetic energy (e.g. heat) supplied to the system is sufficient. The events of protein unfolding are consistent regardless of temperature, however, the rate at which unfolding can occur is highly dependent on the amount of heat supplied [13]. The results of *in vitro* refolding studies have identified that, after the native conformation is lost, a protein may transition between many possible non-native intermediate states. However, energy restraints and the loss of conformational entropy

favour the formation of certain intermediates [14]. These intermediate states are often partially folded, but have regions of unstructured polypeptide backbone and exposed hydrophobicity [15]. The packaging together of these exposed hydrophobic regions on non-native proteins and the consequent formation of oligomeric aggregates is thermodynamically favourable. Protein aggregation is also promoted by the crowded nature of the intracellular environment, which influences the kinetics and equilibria of isomerization and protein association reactions [16]. The aggregates formed may be amorphous, or have an ordered arrangement such as in amyloid fibrils, and eventually reach sizes that exceed their solubility limit [17]. Under certain conditions, non-native proteins may be rescued and refolded back into their native conformation by chaperones (Figure 1).

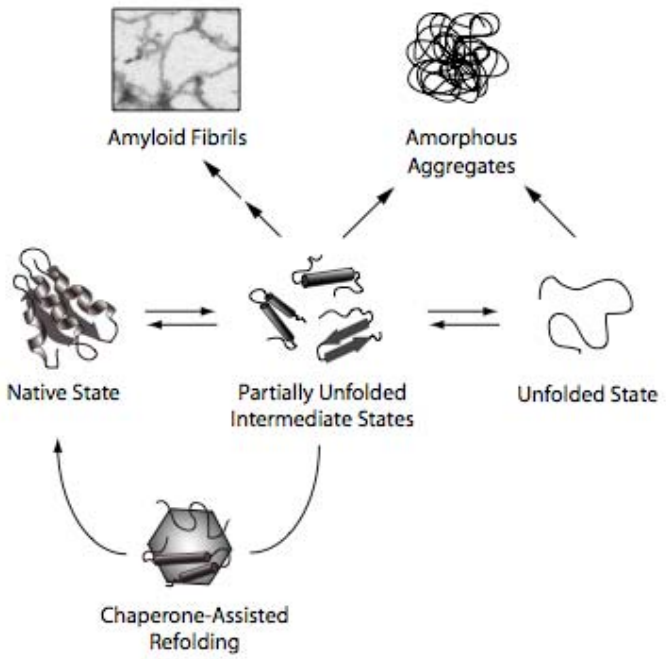

Figure 1. A schematic representation of the protein unfolding and aggregation pathways. When exposed to physical or chemical stresses that destabilize protein structure, a protein may traverse the protein unfolding pathway. This process begins when the protein loses its native three-dimensional structure and adopts a partially unfolded intermediate conformation. Further destabilization of the protein may lead to complete unfolding. However, often before a protein can fully unfold, interactions between adjacent partially unfolded proteins may occur, leading to the formation of either ordered fibrils (e.g. amyloid) or disordered (amorphous) aggregates. To prevent this, chaperones bind partially unfolded 'substrate' proteins and in some cases can facilitate their refolding to a native conformation.

PROTEIN DEPOSITION DISEASES (PDDs)

Controlled unfolding is important in many biological processes including protein translocation, degradation by proteases and regulation of enzyme activity. Additionally, it has been proposed that proteins are dynamic and may constantly transition between less ordered forms and the native conformation [18]. However, uncontrolled unfolding or misfolding and the consequent accumulation of protein aggregates are implicated in the pathology of many diseases collectively known as Protein Deposition Diseases (PDD) (Table 1). PDDs are typically late-onset [19], suggesting that the underlying cause of the disease may be disruption or overwhelming of protein folding quality control mechanisms that were once able to maintain existing proteins in their native conformation. Although the reasons for the progressive impairment of fundamental physiological processes in aging is not fully understood, it is likely that the combination of declining protein folding quality control and exposure to thermal, ionic, heavy metal or oxidative stress may be responsible for late-onset PDDs.

Table 1 Examples of Protein Deposition Diseases (PDDs) and the proteins implicated in their pathology [20-25].

Disease	Protein	Type of Aggregate	Location
Alzheimer's Disease	Amyloid-β	Amyloid	Extracellular
Parkinson's Disease	α-Synuclein	Fibrillar non-amyloid (Lewy Bodies)	Intracellular
Type II Diabetes	Human islet amyloid polypeptide	Amyloid	Intracellular and Extracellular
Amylotrophic Lateral Sclerosis	Superoxide dismutase 1	Fibrillar non-amyloid	Intracellular
Haemodialysis-related Amyloidosis	β_2-Microglobulin	Amyloid	Extracellular
Reactive Amyloidosis	Amyloid-α	Amyloid	Extracellular
Huntington's Disease	Huntingtin	Fibrillar non-amyloid	Intracellular
Creutzfeldt-Jakob Disease	Prion protein	Amyloid	Extracellular
Primary Systemic Amyloidosis	Immunoglobulin light chain	Amyloid	Extracellular
Secondary Systemic Amyloidosis	Serum amyloid-β	Amyloid	Extracellular
Hereditary non-neuropathic systemic amyloidosis	Lysozyme	Amyloid	Extracellular
Corneal dystrophy	Kerato-epithelin	Amorphous	Extracellular
Nonamyloidotic monoclonal IgG deposition disease	Immunoglobulin G	Amorphous	Extracellular
Age-related macular degeneration	62 different proteins	Amorphous (Drusen)	Extracellular
Renal Disease	Tamm-Horsfall protein Osteopotin	Amorphous	Extracellular
Hereditary Renal Amyloidosis	Fibrinogen	Amyloid	Extracellular

Types of Protein Deposits

All PDDs involve protein misfolding leading to the deposition in tissues of insoluble protein aggregates, however, the type of aggregate formed varies between the individual diseases. In many PDDs including Alzheimer's disease, type II diabetes, systemic amyloidosis and transmissible spongiform encephalopathy, the deposition of insoluble aggregates occurs as fibrillar amyloid. The structure and mechanisms underlying the generation of amyloid are discussed elsewhere [26]. In other PDDs the nature of the protein deposits is fibrillar, but not amyloid. Non-amyloid fibrillar structures include Lewy bodies, which are found in Parkinson's disease, Alzheimer's disease, some other forms of dementia and occasionally Shy-Drager's syndrome. Lewy bodies generally consist of a dense core of filaments and granular material radially surrounded by additional filamentous structures. However, randomly arranged Lewy body filaments have been identified in the cerebral cortex of dementia patients [27]. In Pick's disease, non-amyloid fibrillar deposits known as Pick bodies accumulate in specific regions of the central nervous system and contribute towards neuronal damage [28]. Pick bodies consist of randomly oriented straight filaments and periodical, paired, twisted filaments of abnormal tau protein [29].

Amorphous, non-filamentous extracellular aggregates formed by IgG light chain and/or IgG heavy chain are characteristic of nonamyloidotic monoclonal IgG deposition disease (NAMIDD) [30, 31]. The clinical manifestations of NAMIDD are similar to those of amyloid-forming IgG deposition disease and include glomerulonephritis as result of an organ-compromising accumulation of IgG in the kidneys. Pathologic nonamyloid

deposits have also been identified in certain corneal dystrophies. In granular Groenouw type I corneal dystrophy, the progressive accumulation of non-amyloid deposits contributes to corneal opacity and the loss of vision [32]. In Avellino corneal dystrophy both amyloid and non-amyloid material are found co-localized in the cornea [32]. Drusen are amorphous extracellular deposits that accumulate in patients with age-related macular degeneration. In healthy eyes drusen are not found in the macula, however they may exist in the retinal periphery and their size and number are considered a risk factor for developing age-related macular degeneration later in life [33]. Many different proteins are found in drusen with crystallins, lactoglobulin, clusterin, complement component 9, serum albumin, haemoglobin and vitronectin being some of the most abundant and common [34].

Oxidized IgG aggregates are found in many acute and chronic inflammatory states including rheumatoid arthritis [35]. These diseases may be considered a special class of PDDs since oxidized IgG may persist in extracellular fluids as soluble aggregates. IgG aggregates have immune complex-like properties and stimulate neutrophils to release ROS thereby inducing the aggregation of previously unaggregated IgG [36]. This self-perpetuating cycle of neutrophil activation, oxidized IgG aggregation and the generation of damaging ROS is likely to be very important in the pathology of rheumatoid diseases.

Cytotoxicity

While the cytotoxicity of protein aggregates is the focus of many studies, it is unknown whether the deposition in tissues of protein aggregates is, in fact, the critical event in

pathogenesis. Temporal studies of transgenic animals that express abnormal aggregating human proteins have not been very successful at identifying the precise pathogenic species [37]. In diseases such as non-neurological systemic amyloidoses, it appears likely that the large quantity of protein aggregates deposited in tissues cause the clinical symptoms of the disease [20, 21, 23-25]. However, at least in the case of some amyloid diseases there is increasing evidence to suggest that the toxic species may occur early in the aggregation prior to the formation of mature fibrils. One study of amyloid formation has reported that amyloid-beta peptide (Aβ), which is important in Alzheimer's disease pathogenesis, was cytotoxic once amyloidogenic fibrils were formed, but not while it was present as an early-stage amorphous aggregate [38]. Conversely, a study of transgenic mice expressing different mutant forms of Aβ precursor protein showed that behavioural and cognitive defects in the mice preceded amyloid plaque formation [39]. Similarly, evidence of tissue damage before amyloid fibrils were formed was documented in a study of transgenic mice expressing human islet amyloid polypeptide, which is relevant in type II diabetes [40]. Another recent report provides evidence that several amyloid forming proteins are cytotoxic during the early stages of aggregation, but that the mature aggregates themselves are not cytotoxic [41]. If end-point aggregates are not cytotoxic then it is possible that in some cases their formation may be cytoprotective. In particular the intracellular localization of unfolded proteins may increase the efficiency of their autophagic capture and subsequent proteolytic degradation.

Perhaps the most popular theory regarding the cytotoxicity of protein aggregates involves the production of ROS. It has been suggested that cumulative oxidative damage may be

responsible for many PDDs including Alzheimer's disease [42] and age-related macular degeneration [43]. While oxidative stress is a known factor contributing to the unfolding and aggregation of proteins, there is increasing evidence that the accumulation of protein aggregates causes elevated ROS [44-46]. The mechanism by which this occurs remains unclear, however, it may involve the disruption of ion gradients across cell membranes. For example, pre-fibrillar amyloid aggregates share certain structural similarities with other membrane pore-forming proteins and consequently the formation of unregulated ion channels has been proposed as a possible mechanism for their cytotoxicity [47-49]. One suggestion is that ROS are elevated as a result of increased oxidative metabolism to produce adenosine triphosphate (ATP) needed for pumping excess calcium out of the cells [50]. Alternatively, it has been proposed that the Aβ peptide interacts directly with metal ions such as Cu^{2+} and Fe^{3+} (which are found at particularly high levels in amyloid plaques [51]) to produce damaging H_2O_2 [52]. As previously mentioned, a likely possibility for the generation of extracellular ROS in rheumatoid arthritis is stimulation of neutrophils by aggregated IgG [36]. Similarly, the activation of microglia by amyloid-forming Aβ or prion peptides and the subsequent generation of ROS have been implicated in neurodegenerative diseases [53-55]. The release of cytokines, such as IL-6 and IL-1β, have also been linked to neuronal damage [54]. Moreover, minocycline (an inhibitor of microglia activation) was found to be neuroprotective in a murine model of Parkinson's disease [56]. While autoantibodies against amyloid forming structures have been found in Alzheimer's patients [57] it is unclear whether classical autoimmunity contributes to the pathology of this disease, although this has been suggested for other PDDs including age-related macular degeneration [58] and renal disease [59].

Whether the causes of protein aggregate toxicity in individual diseases relate to physical organ/tissue disruption resulting from the deposition of large insoluble aggregates, or to the cytotoxic effects of smaller soluble oligomeric aggregates, to develop new therapies, strategies will need to be adopted which target the relevant underlying molecular mechanism(s). Regardless of the mode of toxicity, it appears likely that PDDs arise when normally efficient protein folding quality control mechanisms are overwhelmed [60]. Therefore, increasing our understanding of extracellular protein folding quality control will be very important in efforts to combat these diseases.

INTRACELLULAR PROTEIN FOLDING QUALITY CONTROL

Quality control of the correct transcription of RNA and translation of the polypeptide sequence is extremely important for ensuring the adoption of correct native protein conformation. The exonuclease activity of DNA polymerase and the proof-reading activity of tRNA are the first lines of defence. However, mistakes in transcription or translation are not always rectified and environmental conditions may promote the partial unfolding of correctly folded proteins. Under these circumstances, the cells post-translational quality control system may rescue abnormal proteins or target them for destruction. Molecular chaperones are central to refolding pathways but also play an important role in the targeting of non-native proteins to the proteasome or lysosomes for degradation. Together these processes act to compensate for the relative instability of proteins, generally preserving the current pool of proteins in their native conformations. Extracellular proteins exit the cell after folding, maturation and modification in the

endoplasmic reticulum (ER). As it is an important site of protein synthesis and trafficking, the ER possesses extensive protein folding quality control machinery. Once polypeptides reach the ER, mistakes in folding or processing are recognized by an intricate system of chaperones and resident proteases. If non-native proteins accumulate, an unfolded protein response may be elicited which ultimately results in increased expression of molecular chaperones and translocation proteins that either rescue and refold the unfolded proteins or direct them to sites of intracellular proteolysis [61]. Non-native proteins destined for secretion that evade the ER quality control may be recognized as abnormal after reaching the golgi apparatus. This results in retrograde transport of the protein back to the ER or re-targeting of the protein to a lysosome for degradation [62]. Together these actions ensure that only correctly folded proteins are secreted from normal healthy cells. However, little is known regarding corresponding mechanisms that may prevent the accumulation of unfolded proteins in extracellular spaces.

EXTRACELLULAR PROTEIN FOLDING QUALITY CONTROL

A 70 kg human contains around 5 L of blood and 10 L of other extracellular fluids including interstitial fluid, cerebrospinal fluid and intraocular fluid. Whole plasma contains around 7.5 % protein (by mass) [63]. This includes secreted proteins and proteins that may have been shed from the cell surface or lost from damaged tissues. Although a small number of proteins make a relatively large contribution to the extracellular protein pool, it has been suggested that over 10,000 different proteins may be normally present in plasma at low levels [64]. In the extracellular environment proteins are exposed to various stresses that may induce partial unfolding; this includes

the hydraulic force of plasma being pumped around the body - hydrodynamic shear-stress is known to contribute to protein unfolding [12, 65]. Additionally, the extracellular environment is relatively more oxidising compared to the cytosol [66]. Increased plasma protein oxidation (as determined by measuring protein carbonyl formation) is characteristic of many disease states including Alzheimer's disease [67], coronary artery disease [68] and uremia [69]. Additionally, the most abundant blood protein, human serum albumin, is known to be vulnerable to damage by ROS [69, 70].

To date, little is known about what (if any) specific mechanisms for protein folding quality control operate in extracellular space. However, evidence has been gathered suggesting that they are likely to exist. This includes the observation that unfolded proteins are degraded more rapidly than native proteins *in vivo* [71]. Additionally, polymorphonuclear leukocytes selectively catabolise denatured proteins compared to native proteins [72] and lysosomal enzymes are implicated in this process [73]. Furthermore, liposomes with exposed surface hydrophobicity are cleared from circulation more rapidly than those with a hydrophilic outer layer [74] and certain modifications of human serum albumin, including oxidation and mutations that increase surface hydrophobicity, enhance albumin clearance from circulating blood [75]. These findings suggest that exposed hydrophobicity may target extracellular molecules for clearance and/or degradation.

There is little evidence to support the existence of an extracellular proteolysis system with specificity for unfolded proteins. While the concentration of plasma proteasome has

been measured at between 2.1-2.4 μg/mL [76], this is 300 times less abundant than inside cells [77]. Moreover, proteasomal degradation requires ATP which is around 1000 times less abundant extracellularly [78]. Considering these limitations it is unlikely that a system of this kind could participate significantly in extracellular protein folding quality control. Intracellular chaperones may be released into extracellular fluids (e.g. after necrotic cell death). While this may have some importance *in vivo*, it is probable that in the instance of mass presentation of partially unfolded protein, such as might be encountered during chronic inflammation, the chaperone capabilities of normally intracellular chaperones (e.g. Hsp70 present in plasma at < 10 ng/mL [79]) would be quickly overwhelmed. Furthermore, intracellular chaperones require ATP to effect protein refolding which as previously mentioned is scarce in extracellular fluids.

A MODEL FOR EXTRACELLULAR PROTEIN FOLDING QUALITY CONTROL

The discovery of three abundant extracellular proteins with chaperone activity is an important landmark in understanding extracellular protein folding quality control. While clusterin, haptoglobin and α_2-macroglobulin do not appear to possess independent protein refolding activity, their ability to stabilize stressed (partially unfolded) proteins is likely to be important in preventing potentially pathological protein deposition [4-6, 80-82]. Several lines of evidence, reviewed in [26], support a role for ECs in the *in vivo* clearance of aggregation-prone extracellular proteins:

1. ECs inhibit stress-induced protein aggregation in unfractionated human plasma, and are found complexed with soluble amyloid-forming proteins in human extracellular fluids.

2. Cell surface receptors are known for all three ECs, and all ECs have previously been implicated in ligand transport across plasma membranes.

3. ECs are associated with extracellular protein deposits in numerous diseases, which may be indicative of a failure in or the overwhelming of the machinery responsible for quality control of extracellular protein folding.

The available evidence supports a model in which ECs bind to regions of exposed hydrophobicity on extracellular stressed proteins to form soluble complexes which are subsequently internalized via receptor-mediated endocytosis. Once internalized, the complexes are most likely disposed of by proteolytic degradation within lysozomes. This model is schematically represented in Figure 2. This mechanism is likely to have profound effects on the development of many diseases for which pathology arises from extracellular protein aggregation (see PROTEIN DEPOSITION DISEASES, above).

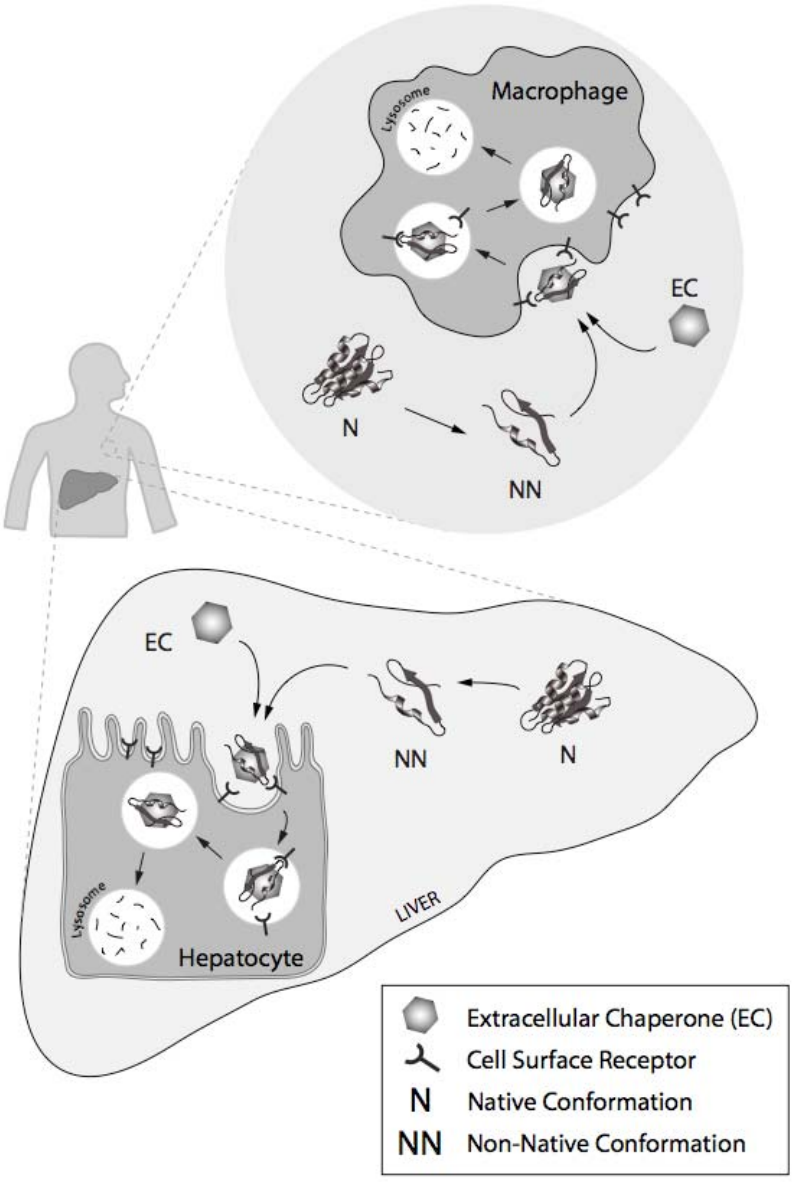

Figure 2. Proposed model of extracellular chaperone-assisted clearance of aberrantly folded proteins. Extracellular chaperones (ECs) bind extracellular proteins in non-native (NN) conformations and facilitate their uptake into cells via receptor-mediated endocytosis. Once internalised, the EC-NN complexes are transported by vesicles towards the lysosome (L), where they are degraded. During this process, the receptor is recycled back to the cell surface. Sites which may be of particular importance in this system are the liver and macrophages.

POTENTIAL DRUG TARGETS

The discovery of components of a system for extracellular protein folding quality control provides an opportunity to identify novel drug targets for those pathological mechanisms that are out of reach of the intracellular protein folding quality control system. Examining the pathways involved in extracellular protein quality control, a number of possible targets are revealed. These include influencing protein aggregation with chemical or peptidic chaperones, and increasing the expression of ECs and endocytic receptors (Figure 3), discussed below.

Inhibition of Aggregate Formation and Toxicity by Chemical and Peptidic Chaperones

Like their molecular chaperone counterparts, a wide range of naturally-occurring or synthetic organic compounds have been reported to inhibit the aggregation/fibrillogenesis of numerous PDD-causing proteins. In some instances, they have also exhibited the potential to reduce aggregate-mediated cellular toxicity. Owing to their chaperone-like properties, these small molecules have been termed “chemical chaperones” [83]. Chemical chaperones have been envisaged to modulate disease progression by reducing aggregate formation leading in turn to decreased protein deposition, aggregate-mediated cytotoxicity and inflammation. Inhibition of protein aggregation by chemical chaperones may be achieved via one or more mechanisms: (i) steric hindrance of protein-protein interaction, (ii) shielding regions of exposed hydophobicity or, (iii) in the case of amyloid fibrillogenesis, minimizing structural changes leading to β-sheet formation.

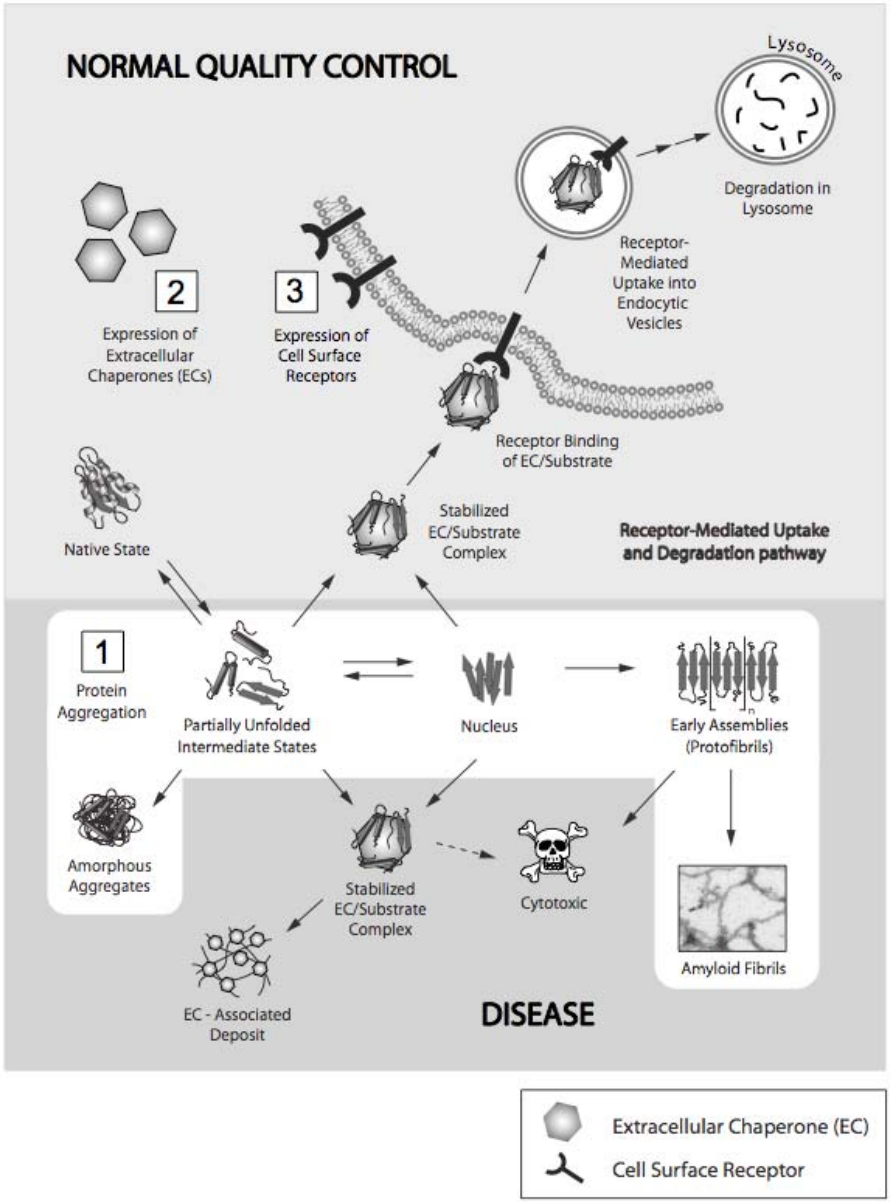

Figure 3. Potential therapeutic targets for inhibiting protein aggregate formation and related disease progression. The available evidence suggests that quality control mechanisms exist *in vivo* to ensure that partially unfolded extracellular proteins do not accumulate in tissues and organs (see Figure 2). Failure of this system may lead to the onset of disease caused by one or both of the following: (i) the accumulation of aberrantly folded assemblies such as insoluble amorphous aggregates, EC-associated deposits, or

degradation-resistant amyloid fibrils, or (ii) cytotoxic effects of pre-fibrillar species generated via the amyloid assembly pathway. Potential targets for therapeutic interventions are (1) protein aggregation itself (whether it leads to amorphous or amyloid deposits), which can be suppressed with small molecule inhibitors, such as chemical chaperones and peptidic inhibitors, and overexpression of (2) extracellular chaperones or (3) endocytic receptors. The latter two effects may enhance the uptake of EC-substrate complexes, thereby increasing the rates of clearance and degradation of potentially pathogenic non-native extracellular proteins.

Molecules such as Congo red and many benzofuran-based compounds have been shown to reduce Aβ-mediated cytotoxicity by preventing Aβ fibrillogenesis or via direct interactions with the soluble Aβ oligomers [38, 84, 85]. In addition, Congo red significantly reduced the cellular accumulation of amyloid-forming prion protein (PrP) in scrapie-infected mouse cells [86]. Other small molecules such as nicotine can also prevent fibrillogenesis of Aβ by enhancing helix stability and preventing the α-helix to β-sheet conversion required for Aβ polymerization [87].

Osmolytes increase the thermodynamic stability of proteins by reducing the exposure of their hydrophobic regions to solvent [88-90]. This promotes a hydrophobic collapse in the unfolded protein leading to a rapid equilibrium shift towards a more stable conformation. Exemplifying this, the osmolyte trimethylamine-N-oxide (TMAO) can force highly unstructured proteins to fold into conformations that are native-like in conformation [88]. Moreover, TMAO and other osmolytes including glycerol, dimethyl sulfoxide (DMSO) and proline are reportedly able to assist the processing of disease-related mutant proteins [91, 92] and prevent their aggregation [93]. Despite showing

much promise, compared to protein chaperones, the lack of specificity and the high (micromolar) concentrations required limit the use of chemical chaperones as effective therapeutics in humans.

To overcome the limitations of chemical chaperones, several groups have investigated the possible use of small peptides to inhibit protein aggregation and/or to reduce aggregate-mediated cellular toxicity. The advantages of using peptidic inhibitors, as opposed to non-peptidic compounds, include high efficacy and specificity. One approach utilises the dual roles of 'hybrid peptides'. These peptides typically contain a "recognition" domain, for specific binding to target protein/s, and a "disrupting" domain, which interferes with the protein aggregation event. In general, the recognition domain usually corresponds to the region(s) of the target protein identified as being critical for its aggregation/fibrillogenesis. Once bound to the target protein, the disrupting domain modulates protein aggregation via one of several mechanisms (e.g. steric hindrance, charge repulsion, hydrophobicity) depending on the nature of the domain. The success of this strategy has so far been demonstrated for Aβ [94] and insulin [95].

Another approach for preventing aggregate formation focuses on the ability of some peptides to interfere with β-sheet formation and the stacking processes that is crucial for amyloid fibril formation [96]. This strategy relies on three major properties of 'β-sheet breaker' peptides (i) to ensure specific binding β-sheet breakers are usually derived from the amyloidogenic sequences of their intended protein targets, (ii) known β-sheet-interrupting amino acid residues, such as proline, are incorporated [97] and (iii) charged

residues are often added to either end of the peptide to improve solubility [98]. β-sheet breaker peptides have been shown to be effective in disrupting amyloid formation by Aβ [96, 99, 100], PrP [101], and the yeast prion protein Sup35 [102]. Although there is no precedent for this in the literature, any small molecule approach directed towards inhibiting extracellular protein aggregation and/or toxicity might conceivably also affect receptor-mediated clearance processes. Thus, each potential therapeutic molecule would need to be examined for such effects as part of characterizing it *in vivo* efficacy.

Inducing Expression of Extracellular Chaperones and Their Receptors

In vitro, at very low molar ratios of clusterin:substrate, clusterin enhances amyloid formation, but has the opposite effect when present at higher but still sub-stoichiometric ratios [103]. Moreover, complexes of clusterin and Aβ formed at relatively high ratios of clusterin:Aβ (e.g. 1:10) were less toxic to neuroblastoma cells than Aβ alone; in contrast, complexes formed at low ratios of clusterin:Aβ (e.g. 1:500) were more toxic than Aβ alone [103]. This suggests that increasing the levels of clusterin, and possibly other ECs, would be protective *in vivo*. In fact, data from *in vivo* models suggest this is the case (reviewed in [26]). Collectively, these data suggest that overexpression of ECs may prove beneficial in PDDs such as Alzheimer's disease. One potential route of therapy would be to use small molecules to induce higher expression of ECs. Small molecules have been found that can upregulate the heat shock response, subsequently increasing the concentration of intracellular chaperones. One such molecule, arimoclomal, binds to heat shock factor-1 (HSF-1) to prolong its activation [104] - this drug has been shown to extend the lifespan of mice in a model of the PDD amyotrophic lateral sclerosis [105].

There has also been progress made through the use of high throughput screens of hundreds of compounds to identify celastrol, a natural product from the *Celastraceae* family of plants, as a heat shock inducer [106]. This may also be a useful approach in the case of clusterin as it has an element in its promoter region that responds to HSF1 and HSF2 [107]. Other pathways have also been indentified that control expression of these ECs; the promoter regions of the clusterin, haptoglobin and α_2-macroglobulin genes contain AP-1 (activator protein-1) recognition motifs [108-110]. Thus the AP-1 pathway provides additional targets for inducing EC expression.

However, increasing the expression of ECs may also have detrimental side effects. Increased levels of clusterin are known to be associated with cancer progression and clusterin is known to protect cells from chemotherapy drugs such as paclitaxel [111]. Moreover, increasing the levels of soluble chaperone-stressed protein complexes, without increasing the throughput of pathways that would remove them, may increase toxicity. A number of lines of evidence suggest that increasing the expression of receptors mediating endocytosis of EC-stressed protein complexes may be a necessary adjunct to inducing EC expression. For example, while α_2-macroglobulin protected neuroblastoma cells expressing LRP from Aβ toxicity, it did not protect cells lacking LRP [112]. In this case the protective effect of α_2-macroglobulin could be inhibited by RAP (a pan-specific inhibitor of LRP ligand binding). In addition, α_2-macroglobulin has been shown to promote Aβ toxicity towards LRP-negative LAN5 cells but has the opposite effect with LRP-expressing LAN5 transfectants [112]. Furthermore, in Alzheimer's disease patients there are increased plasma concentrations of several LRP ligands including ApoE, α1

anti-chymotrypsin and urokinase [113-115]; this raises the possibility that in Alzheimer's disease LRP may be either overwhelmed or its expression downregulated. This has been confirmed by data showing that LRP expression at the blood brain barrier is decreased in Alzheimer's disease patients and transgenic Aβ-overproducing mice [116].

It is important to note that a strategy of inducing the expression of receptors mediating the uptake of EC-stressed protein complexes may be complicated by the fact that receptor expression is controlled by signalling pathways also associated with the activation of immune cells [117, 118]. Thus, using small molecule transcriptional activators to increase the expression of the relevant cell surface receptors is likely to activate cells of the innate immune system such as macrophages. Acute activation of cells such as microglia in the Alzheimer's brain [119] and Creutzfeldt-Jakob disease [120], and neutrophils in rheumatoid arthritis [121] are thought to contribute to pathology by the release of toxic mediators such as reactive oxygen species (ROS) and proinflammatory cytokines [122, 123].

Thus, the available evidence suggests that it may be quite difficult to identify and/or design small molecules that increase the transcription of ECs and their receptors without inducing unknown, possibly detrimental, side effects. Despite these complexities, ECs remain an attractive target for potential therapeutics in PDDs, which are thought to arise as a result of an age-dependent overloading or malfunction of the protein folding quality control systems [124]. One potential way of controlling the expression of ECs and their receptors, without affecting the expression of other genes, is by targeting their translation

rather than transcription. Recent work has shown that a small molecule designed to target a site in ferritin mRNA can induce an increase in the rate of its translation [125]. Thus by specifically targeting the mRNA of specific ECs and their receptors it may be possible to eliminate the problem of the non-specific upregulation of a range of genes, controlled by the promoter elements targeted. In order to reach the goal of using small molecules to regulate elements of the extracellular protein folding quality control system, our knowledge of this system needs to be increased. This expanded knowledge base will become critically important as the population of the world continues to age resulting in extracellular PDDs placing an ever increasing burden on society.

CONCLUDING REMARKS

Many serious human diseases, collectively known as the PDDs, have pathologies that arise from the inappropriate aggregation and deposition of partially unfolded proteins. In many cases, for example Alzheimer's disease and prion diseases, these processes occur extracellularly. Although the mechanisms that act to control protein folding intracellularly are relatively well understood, corresponding processes that operate extracellularly have only recently begun to be identified. Abundant ECs and endocytic cell surface receptors are likely to play important roles in clearing partially unfolded extracellular proteins and preventing them causing pathologies. This new understanding has broadened the potential therapeutic targets for PDDs to include, in addition to chemical/peptidic chaperones, manipulation of the expression levels of ECs and endocytic receptors.

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